

Nucleic Acid Molecules Encoding Polypeptides Having The Enzymatic Activity Of An RNA-directed RNA Polymerase (RdRP)

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SUMMARY OF THE INVENTION

The present invention relates to nucleic acid molecules encoding polypeptides having the enzymatic activity of an RNA-directed RNA polymerase (RdRP). The present invention also provides vectors comprising said nucleic acid molecules, wherein the nucleic acid molecules are operatively linked to regulatory elements allowing expression in prokaryotic and/or eukaryotic host cells. Additionally, the present invention relates to polypeptides encoded by said nucleic acid molecules and methods for the production of said polypeptides. The present invention further relates to pharmaceutical and diagnostic compositions as well as kits comprising the aforementioned nucleic acid molecules and/or comprising a nucleic acid molecule which is complementary to such a nucleic acid molecule. Said compositions and kits may further comprise polypeptides encoded by the described nucleic acid molecules. Furthermore, the present invention relates to antagonists and inhibitors of the aforesaid polypeptides and/or antibodies specifically recognizing such polypeptides. Also within the scope of the present invention are methods and uses comprising the nucleic acid molecules, vectors, polypeptides, antibodies and antagonists and inhibitors of the invention for modulating gene expression in humans and animals. Furthermore, the present invention relates to transgenic plant cells and plants containing the aforementioned nucleic acid molecules as well as the use of the aforementioned nucleic acid molecules, polypeptides and/or antagonists/inhibitors in plant cell culture and plant tissue culture and/or plant breeding.

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BACKGROUND OF THE INVENTION

~~Background of the invention~~

Since the development of plant transformation systems a particular interest exists on the stable expression of foreign genes in higher plants. Although these attempts were successfully realized for several herbicide resistance genes, a series of experiments failed that had been designed to use plants as efficient bio-reactors. It seemed that higher plants have a system of defense to protect themselves against overexpression of foreign genes. Whenever a transgene expression comes up to a threshold dose it is without selective pressure silenced either by transcriptional or post-transcriptional inactivation. Recent studies on these phenomena indicated that "antisense" RNA might be responsible for these silencing mechanisms. The idea of an "antisense" RNA-mediated gene silencing was substantiated by the analysis of transgenically mediated virus resistance in plants (Smith *et al.*, 1994; English *et al.*, 1996; Sijen *et al.*, 1996). It could be shown that the *in vivo* transcripts of non-translatable "sense" cDNA constructs which had been integrated into the plant genome can mediate resistance against infection with plant viruses. Further analysis revealed that this resistance which was due to post-transcriptional gene silencing (PTGS) only occurred when the transgene shared homology with the infecting virus. Based on these data and with regard to previous results describing the co-suppression phenomena in plants (for review see Meyer, 1996) several models explaining the PTGS mechanism have been introduced (English *et al.*, 1996; Baulcomb, 1996; Sijen *et al.*, 1996). Common to all these models is the assumption that "antisense" RNAs are synthesized from "sense" RNA templates by an RNA-directed RNA polymerase (RdRP). Subsequently the produced "antisense" RNAs can hybridize to complementary parts of mRNAs or invading virus RNAs which would unresistingly lead to degradation of the double-stranded regions.

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It is most likely that this PTGS is not restricted to transgenes but that it is also involved in normal plant gene regulation. Moreover, it is most likely that the same mechanism takes place in all higher eukaryotes. Therefore, a detailed examination of RNA-mediated gene regulation had become an important aspect of basic and applied research. An RNA-dependent RNA polymerase (EC 2.7.7.48, RdRP) activity has been detected in healthy plant tissue, for review see Fraenkel-Conrat (1986). Previous studies of RdRP activity have suffered from the inhomogeneity of enzyme preparations and of the resulting RNA products in that they did not allow the precise determination of their template complementarity by direct RNA sequencing. Schiebel et al. (1993 a, b) provided evidence for the concept that the RdRP mediated transcription is a truly RNA-instructed process yielding products that are precise complementary copies of the RNA template offered to an RdRP active enzyme preparation. However, although purification of the RdRP from tomato leaf tissue to electrophoretic homogeneity was reported (Schiebel et al., 1993 a, b), the enzyme preparations were not approachable for amino acid sequencing. One reason for the slow progress in elucidating the amino acid sequence of the RdRP is to be seen in the fact that only low amounts of protein could be isolated using the hitherto available technology and/or that there existed further proteins in the enzyme preparations obtainable with standard techniques which resulted in non-informative or even false sequence information. However, for efficient use of RdRP associated technology, it was desirable to be able to manipulate the genetic material associated with said technology.

Thus, the technical problem underlying the present invention is to provide such material.

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A **DETAILED DESCRIPTION OF THE INVENTION**
1 ~~Description of the invention~~

The solution to the technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the invention relates to a nucleic acid molecule encoding a polypeptide having the enzymatic activity of an RNA-directed RNA polymerase (RdRP) or encoding an enzymatically active fragment thereof selected from the group consisting of:

- (a) nucleic acid molecules encoding a polypeptide comprising the amino acid sequence given in SEQ ID NO:2;
- (b) nucleic acid molecules comprising the nucleotide sequence given in SEQ ID NO:1;
- (c) nucleic acid molecules hybridizing with a complementary strand of a nucleic acid molecule as defined in (a) or (b); and
- (d) nucleic acid molecules, the nucleotide sequence of which is degenerate as a result of the genetic code to a nucleotide sequence of a nucleic acid molecule as defined in (c).

In accordance with the present invention, a nucleic acid molecule encoding a novel class of RdRPs has been identified. This has been achieved by using a novel purification method for the isolation of a polypeptide having the enzymatic activity of an RdRP which was suitable for amino acid sequencing. Oligonucleotides were designed and used for cloning of the corresponding cDNA.

The polypeptide encoded by the nucleic acid sequence of SEQ ID NO: 1 from nucleotide 194 to nucleotide 3535 encodes a polypeptide of a 1114 amino acids with a calculated molecular weight of about 127 kDa. This data is in good agreement with the prior art experimental values that had been determined by SDS-PAGE (128 kD) and sucrose gradient centrifugation (119 kD) (Schiebel et al., 1993a). The nucleotide and the amino acid sequence

given in SEQ ID NOS: 1 and 2, respectively, displayed no significant homologies to sequences in any database (Blast X, GAP, Wisconsin Sequence Packaging System, Version 7.0) searched. In addition, the presence of putative phosphorylation sites for putative protein kinases can be identified by computer aided search for phosphorylation consensus sequences which suggests possible regulation by phosphorylation. Studies which had been carried out in accordance with the present invention revealed that the polypeptide encoded by the nucleotide sequence of SEQ ID NO:1 is identical with the RdRP isolated from tomato plants as described in example 1 of the present application.

The polypeptide encoded by the nucleotide sequence of SEQ ID NO:1 displays the ability to catalyze *in vitro* transcription of preferably short single-stranded RNA and DNA molecules into precisely complementary RNA copies up to the full length of these templates. The RdRP-directed transcription can be primed by RNA and DNA oligonucleotides complementary to the 3'-terminal nucleotides of the template. In addition, the RdRP encoded by the nucleic acid molecule of the invention can catalyze unprimed transcription. An unprimed transcription starts preferentially at the 3'-terminal nucleotides of a corresponding template. Furthermore, the RdRP is capable of adding a single noncomplementary nucleotide to the 3' terminus; see also Schiebel et al. (1993b).

The term "RNA-directed RNA polymerase" (RdRP), as used herein, means that said polypeptide or enzymatically active fragment thereof is capable of RNA-directed RNA synthesis, thus using RNA as a template for synthesizing complementary RNA molecules. However, as set forth above, said RdRP may also be capable of accepting single-stranded DNA molecules as templates for RNA transcription.

The availability of the nucleic acid molecules encoding the RdRP is definitely a major advantage because now experiments are possible that allow both the

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"knock-out" and the improvement of the regulation mechanism for a given gene.

The nucleotide sequence depicted in SEQ ID NO: 1, in general, encodes a novel class of polypeptides having RdRP activity. By the provision of this nucleotide sequence, it is now possible to isolate identical or homologous nucleic acid molecules which encode polypeptides with the enzymatic activity of said polypeptide from other species or organisms.

Thus, the invention also relates to nucleic acid molecules hybridizing with the above-described nucleic acid molecules and differ in one or more positions in comparison with these as long as they encode a polypeptide having RdRP activity. Such molecules comprise those which are altered, for example, by deletion(s), insertion(s), substitution(s), addition(s) and/or recombination(s) or any other modification(s) known in the art either alone or in combination in comparison to the above-described nucleic acid molecules. Methods for introducing such modifications in the nucleic acid molecules according to the invention are well-known to the person skilled in the art. The invention also relates to nucleic acid molecules the sequence of which differs from the nucleotide sequence of any of the above-described nucleic acid molecules due to the degeneracy of the genetic code.

By "hybridizing" it is meant that such nucleic acid molecules hybridize under conventional hybridization conditions, preferably under stringent conditions such as described by, e.g., Sambrook et al. (Molecular Cloning; A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)).

In a preferred embodiment the nucleic acid molecules according to the invention are RNA or DNA molecules, and most preferably cDNA or genomic DNA. It should also be understood that the nucleic acid molecules of the invention may be synthetically synthesized DNA or RNA molecules or hybrids

thereof. Preferably, said nucleic acid molecules may be prepared from nucleic acids obtained from any organism, tissue or cell, namely from bacteria, eucaryotes or archaebacteria and preferably from mammalian, plant, insect, bacterial, or fungal cells, worm cells such as derived from *C. elegans* or viruses. In a further most preferred embodiment the nucleic acid molecule of the invention is derived from a plant, preferably from tomato.

Nucleic acid molecules hybridizing with the above-described nucleic acid molecules can, in general, be derived from any organism comprising such molecules, preferably from monocotyledonous or dicotyledonous plants, in particular from any plant of interest in agriculture, horticulture or wood culture, such as crop plants, namely those of the family Poaceae, any other starch producing plants, such as potato, maniok, leguminous plants, oil producing plants, such as oilseed rape, flaxseed, etc., plants using polypeptide as storage substances, such as soybean, plants using sucrose as storage substance, such as sugar beet or sugar cane, trees, ornamental plants etc. Preferably the nucleic acid molecules according to the invention are derived from plants belonging to the family Solanaceae. Identical or similar polypeptides may also be found in animals, preferably mammals. Nucleic acid molecules hybridizing to the above-described nucleic acid molecules can be isolated, e.g., from libraries, such as cDNA or genomic libraries by techniques well known in the art. For example, hybridizing nucleic acid molecules can be identified and isolated by using the above-described nucleic acid molecules or fragments thereof or complementary sequences thereof as probes to screen libraries according to standard techniques. Possible is also the isolation of such nucleic acid molecules by applying the polymerase chain reaction (PCR) using as primer oligonucleotides derived from the above-described nucleic acid molecules.

Nucleic acid molecules which hybridize with any of the aforementioned nucleic acid molecules also include fragments, derivatives and allelic variants of the above-described nucleic acid molecules that encode a polypeptide

having RdRP activity or an enzymatically active fragment thereof. Fragments are understood to be parts of nucleic acid molecules long enough to encode the described polypeptide or an enzymatically active fragment thereof. The term "derivative" means in this context that the nucleotide sequence of these nucleic acid molecules differs from the sequences of the above-described nucleic acid molecules in one or more nucleotide positions and homologous to said nucleic acid molecules. Homology is understood to refer to a sequence identity of at least 40 %, particularly an identity of at least 60 %, preferably more than 80 % and still more preferably more than 90 %. The deviations from the sequences of the nucleic acid molecules described above can, for example, be the result of substitution(s), deletion(s), addition(s), insertion(s) and/or recombination(s) either alone or in combination.

Homology further means that the respective nucleic acid molecules or encoded polypeptides are enzymatically and/or structurally equivalent. The nucleic acid molecules that are homologous to the nucleic acid molecules described above and that are derivatives of said nucleic acid molecules are, for example, by way of variations of said nucleic acid molecules which represent modifications having the same enzymatic function. They may be naturally occurring variants, such as sequences from other plant varieties or species, or obtained by mutation. These mutations may occur naturally or may be obtained by mutagenesis techniques. The allelic variants may be naturally occurring allelic variants as well as synthetically produced or genetically engineered variants.

The polypeptides encoded by the various derivatives and variants of the above-described nucleic acid molecules share one or several specific common characteristics, such as enzymatic activity, molecular weight, immunological reactivity, conformation, etc., as well as physical properties, such as electrophoretic mobility, chromatographic behavior, sedimentation coefficients, pH optimum, temperature optimum, stability, solubility, spectroscopic properties, etc.

All such fragments, variants and derivatives of the RdRP of the invention are included within the scope of this invention, as long as the essential characteristic RdRP activity as defined above remains unaffected in kind.

In a further embodiment, the invention relates to nucleic acid molecules of at least 15 nucleotides in length hybridizing specifically with a nucleic acid molecule as described above or with a complementary strand thereof. This means that they hybridize, preferably under stringent conditions, specifically with the nucleic acid molecules as described above and show no or very little cross-hybridization with nucleic acid molecules encoding other polypeptides. Such nucleic acid molecules may be used as probes and/or for the control of gene expression. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary in length. The nucleic acid probes of the invention are useful for various applications. On the one hand, they may be used as PCR primers for amplification of nucleic acid molecules according to the invention. On the other hand, they can be useful tools for the detection of the expression of nucleic acid molecules according to the invention in plants or any other organism, for example mammals, by e.g. in-situ hybridization or Northern-Blot hybridization. Another application is the use as a hybridization probe to identify nucleic acid molecules hybridizing to the nucleic acid molecules according to the invention by homology screening of genomic or cDNA libraries. Nucleic acid molecules according to this preferred embodiment of the invention which are complementary to a nucleic acid molecule as described above may also be used for repression of expression of a nucleic acid molecule encoding a polypeptide having RdRP activity, for example due to an antisense effect or for the construction of appropriate ribozymes which specifically cleave such nucleic acid molecules. Furthermore, the person skilled in the art is well aware that it is also possible to label such a nucleic acid probe with an appropriate marker for specific applications, such as for the detection of the presence of a nucleic acid molecule of the invention in a sample derived from an organism.

The present invention also relates to vectors, particularly plasmids, cosmids, viruses, bacteriophages used conventionally in genetic engineering that contain a nucleic acid molecule according to the invention.

In a preferred embodiment the nucleic acid molecule present in the vector is linked to regulatory elements which allow the expression of the nucleic acid molecule in prokaryotic and/or eukaryotic cells. The nucleic acid molecule encoding the polypeptide having RdRP activity and the template nucleic acid molecule may be contained in different or in the same vector. Expression comprises transcription of the nucleic acid molecule, preferably into a translatable mRNA. Regulatory elements ensuring expression in prokaryotic and/or eukaryotic cells are well known to those skilled in the art. In the case of eukaryotic cells they normally comprise promoters ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript. Additional regulatory elements may include transcriptional as well as translational enhancers.

The present invention furthermore relates to host cells comprising a vector as described above or a nucleic acid molecule according to the invention wherein the nucleic acid molecule is foreign to the host cell.

By "foreign" it is meant that the nucleic acid molecule is either heterologous with respect to the host cell which means that said nucleic acid molecule is derived from a cell or organism with a different genomic background, or that is homologous with respect to the host cell but located in a different genomic environment than the naturally occurring counterpart of said nucleic acid molecule. This means that, if the nucleic acid molecule is homologous with respect to the host cell, it is not located in its natural location in the genome of said host cell. In this case the nucleic acid molecule may be either under the control of its own promoter or under the control of a heterologous promoter. The vector or nucleic acid molecule according to the invention which is

present in the host cell may either be integrated into the genome of the host cell or it may be maintained extrachromosomally. In this respect, it is also to be understood that the nucleic acid molecule of the invention can be used for "gene targeting" and/or "gene replacement", for restoring a mutant gene or for creating a mutant gene via homologous recombination.

The host cell can be any prokaryotic or eukaryotic cell, such as a bacterial, insect, fungal, worm, plant or animal cell. Preferred fungal cells are, for example, those of the genus *Saccharomyces*, in particular those of the species *S. cerevisiae*.

Another object of the invention is a method for the preparation of such polypeptides encoded by the nucleic acid sequence of the invention which comprises the cultivation of host cells according to the invention which, due to the presence of a vector or a nucleic acid molecule according to the invention, are able to express such a nucleic acid molecule, under conditions which allow expression of the nucleic acid molecule and recovering of the so-produced polypeptide from the culture. Depending on the specific constructs and conditions used, the polypeptide may be recovered from the cells, from the culture medium or from both. For the person skilled in the art it is well known that it is not only possible to have as an expression product a native polypeptide but also to obtain the polypeptide as a fusion polypeptide or to add signal sequences directing the polypeptide to specific compartments of the host cell, ensuring secretion of the polypeptide into the culture medium, etc.

The present invention furthermore relates to polypeptides encoded by the nucleic acid molecules according to the invention or produced by the above-described method, and to enzymatically active fragments of such polypeptides having RdRP activity. In this context, it is also understood that the polypeptides according to the invention may be further modified by

In a preferred embodiment the above-described polypeptide or fragment is phosphorylated. As set forth above, the amino acid sequence of the polypeptide of the invention may be phosphorylated due to the presence of possible phosphorylation sites. Thus, it may be possible to regulate the function of said polypeptide by phosphorylation. Phosphorylation of the polypeptide of the invention may be effected *in vivo* in the host which expresses said polypeptide or *in vitro* by, for example, bringing an appropriate protein kinase in contact with said polypeptide or fragment.

Furthermore, the present invention relates to antibodies specifically recognizing polypeptides according to the invention or parts, i.e. specific fragments or epitopes, of such polypeptides. Specific epitopes or fragments may, for example, comprise amino acid sequences which constitute domains which are characteristic for the polypeptides according to the invention, such as described in the appended examples.

These antibodies can be monoclonal antibodies, polyclonal antibodies or synthetic antibodies as well as fragments of antibodies, such as Fab, Fv, or scFv fragments etc. These antibodies or fragments thereof can be used, for example, for the immunoprecipitation and immunolocalization of polypeptides

according to the invention as well as for the monitoring of the synthesis of such polypeptides, for example, in recombinant organisms, and for the identification of polypeptides interacting with the polypeptides according to the invention. They can further be employed in the screening of expression libraries.

Moreover, the present invention relates to an antagonist/inhibitor of the polypeptide of the invention. Potential antagonists are, for example, oligonucleotides or antibodies which bind to a nucleic acid molecule or polypeptide of the invention, respectively, such that either the expression of the nucleic acid molecule encoding a polypeptide of the invention or the RdRP activity of the polypeptide of the invention is prevented. Potential antagonists also include antisense constructs prepared through the use of antisense technology. Another potential antagonist is a small molecule which binds to the catalytic or to a regulatory portion of the polypeptide of the invention such that normal enzymatic activity is prevented. Examples of small molecules include, but are not limited to nucleotide analogs, small polypeptides or peptide-like molecules or any combinations thereof. Such antagonists may be identified by using the nucleic acid molecules, vectors and polypeptides of the invention.

Thus, in a further embodiment the present invention relates to a method for identifying an antagonist/inhibitor of the polypeptide of the invention comprising:

- (a) contacting the polypeptide of the invention with a plurality of compounds to be screened;
- (b) determining whether said polypeptide is still capable of RNA-directed RNA synthesis; and
- (c) identifying the compound which inhibits the RNA-directed RNA synthesis.

Said plurality of compounds may be comprised in, for example, cell extracts from, e.g. plants or microorganisms. Furthermore, said compounds may be known in the art but hitherto not known to be an activator/inhibitor of the polypeptide of the invention. In the later case, the method would comprise the following steps:

- (a) contacting the polypeptide of the invention with a compound to be tested;
- (b) determining whether said polypeptide is still capable of RNA-directed RNA synthesis.

As discussed in the background section of the description of the present invention, the RdRP synthesizes "antisense" RNAs from "sense" RNA-templates. Said "antisense" RNAs can hybridize to complementary parts of mRNAs or invading virus RNAs, which would lead to degradation of the so formed double-stranded regions. Furthermore, said "antisense RNAs" may bind to DNA, for example, via triple helix formation and thereby block gene expression. Thus, it is possible to suppress the expression of target nucleic acid molecules and preferably genes by introduction of template nucleic acid molecules transcribed by RdRP into host cells which either naturally express an RdRP or which were genetically engineered for the expression or overexpression of the polypeptide of the invention. The degree of "antisense" RNA production may depend on said template nucleic acid molecules in serving as an efficient template for the RdRP. With the aid of the RdRP of the present invention, it is now possible to determine the appropriate templates.

Thus, in a further embodiment, the present invention relates to a method for determining whether a nucleic acid molecule is capable of serving as a template for RNA-directed RNA synthesis comprising:

- (a) contacting the polypeptide of the invention with a preferably single stranded nucleic acid molecule; and
- (b) determining whether the complementary strand of said nucleic acid molecule is synthesized.

The term "template" in the sense of the present invention relates to nucleic acid molecules of at least 15 nucleotides in length which are capable of serving as a template for the polypeptide of the invention. In other words said template can be transcribed into complementary RNA copies, preferably up to the full length of these templates. Said template nucleic acid molecule may be single stranded RNA or preferably single stranded DNA or a hybrid thereof. Furthermore, said template nucleic acid molecule may contain, for example, thioester bonds and/or nucleotide analogs commonly used in oligonucleotide antisense approaches. Such modifications may be useful for the stabilisation of the template nucleic acid molecule against endo- and/or exonucleases. The template nucleic acid molecules may also be transcribed by an appropriate vector containing a chimeric gene which allows for the transcription of a template RNA in prokaryotic and/or eukaryotic cell.

Moreover, the present invention relates to a pharmaceutical composition comprising at least one of the aforementioned nucleic acid molecules, vectors, polypeptides, antibodies and/or antagonists/inhibitors according to the invention either alone or in combination, and optionally a pharmaceutically acceptable carrier or excipient. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g. by intravenous, intraperitoneal, subcutaneous, intramuscular, topical or intradermal administration.

Although the RdRP-mechanism so far is only described for plants, it is now possible to employ the nucleic acid molecules, vectors and polypeptides of the invention for the suppression of undesired gene expression in humans

An additional medical application against undesired gene expression, e.g., virus infections and cancer is given when an active RdRP enzyme is produced *in vitro*. There are more and more attempts to cure virus infections and cancer with the aid of the "antisense" RNA-technique in that synthetic "antisense" oligonucleotides are injected into the corresponding cells. This therapy mainly depends on the choice of the oligonucleotide. It is hardly predictable whether chosen oligonucleotides are stable in the cytoplasm of the target cell and whether they are efficiently directed to an accessible region of the target RNA. Moreover high amounts of "antisense" RNAs have to be injected to ensure that the target RNA is completely degraded.

A more promising approach could be performed by activating the endogenous "RdRP-system" with appropriate "sense" RNA if such system exist in the organism. Following this approach there are major advantages. Useful "sense" nucleic acid molecules can be screened for by an *in vitro* RdRP assay as, for example, described above in that the suitability of said molecules in serving as an efficient template for the enzyme is analysed. If a suitable template is isolated, only low copy numbers of the template nucleic acid molecule have to be successfully transferred into the target cells. The endogenous RdRP should amplify the corresponding "antisense" RNA which will subsequently induce post transcriptional gene silencing.

The present invention also relates to diagnostic compositions or kits comprising at least one of the aforementioned nucleic acid molecules, vectors, polypeptides, antibodies and/or antagonists/inhibitors, and in the case of diagnostic compositions optionally suitable means for detection.

The various compounds comprised in the kit and the diagnostic composition of the invention are preferably bottled in different containers.

Said diagnostic compositions may be used for methods for detecting expression of a polypeptide of the invention by detecting the presence of mRNA encoding a polypeptide having RdRP activity which comprises isolation of mRNA from a cell and contacting the mRNA so obtained with a probe comprising a nucleic acid probe as described above under hybridizing conditions, detecting the presence of mRNA hybridized to the probe, and thereby detecting the expression of the polypeptide by the cell.

Further methods of detecting the presence of a polypeptide according to the present invention comprises immunotechniques well known in the art, for example enzyme linked immunosorbent assays.

Moreover, the present invention relates to the use of and methods employing a nucleic acid molecule, vector, and/or polypeptide of the invention and optionally or preferably a template nucleic acid molecule for treating a disease which is caused by the undesired expression or overexpression of a gene.

It is envisaged by the present invention that the nucleic acid molecules and polypeptides are administered either alone or in any combination, and optionally together with an appropriate RdRP template nucleic acid molecule described above, and/or together with a pharmaceutically acceptable carrier or excipient. Subsequent to administration, said nucleic acid molecules may be stably integrated into the genome of the mammal. On the other hand, viral vectors may be used which are specific for certain cells or tissues which persist in said cells thereby conferring expression of the nucleic acid molecules in said cells. Suitable pharmaceutical carriers and excipients are well known in the art. Elements capable of targeting a nucleic acid molecule

and/or polypeptides to specific cells are described in the prior art, for example, Somia et al., Proc. Natl. Acad. Sci., USA 92 (1995), 7570-7574. The pharmaceutical compositions prepared according to the invention can be used for the prevention or treatment or delaying of different kinds of diseases, which are related to the expression or overexpression of a given gene or genes.

Furthermore, it is possible to use a pharmaceutical composition of the invention which comprises a nucleic acid molecule or vector of the invention, and optionally a template nucleic acid molecule in gene therapy. Naturally, both nucleic acid molecules may be also comprised in the same vector. For example, research pertaining to gene transfer into cells of the germ line is one of the fastest growing fields in reproductive biology. Gene therapy, which is based on introducing therapeutic genes into cells by ex-vivo or in-vivo techniques is one of the most important applications of gene transfer. Suitable vectors and methods for in-vitro or in-vivo gene therapy are described in the literature and are known to the person skilled in the art. The pharmaceutical compositions according to the invention can be used for the treatment of kinds of diseases hitherto unknown as being related to the expression and/or over expression of genes.

It is to be understood that the introduced nucleic acid molecules express an enzymatically active polypeptide of the invention after introduction into said cell and preferably remain in this status during the lifetime of said cell. On the other hand, the person skilled in the art may also use the nucleic acid molecules of the invention to "knock out" an endogenous gene encoding an RdRP, for example, by gene targeting or antisense or ribozyme technology.

In a further preferred embodiment of the method of the invention, said cell is a germ line cell or embryonic cell or derived therefrom. In a most preferred embodiment, said cell is an egg cell or derived therefrom.

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In a further embodiment the invention relates to a method for treating a disease caused by the undesired expression or overexpression of a nucleic acid molecule comprising:

- (a) obtaining cells from a mammal;
- (b) introduction of a nucleic acid molecule or a vector of the invention and optionally a template nucleic acid molecule derived from said nucleic acid molecule which causes the disease into said cells; and
- (c) reintroducing the cells obtained as a product of step (b) into said mammal or into a mammal of the same species.

Said template nucleic acid molecule is preferably identified or determined by methods described hereinabove. Preferably, said mammal is a human, rat or mouse and/or the cell is a germ cell, an embryonic cell or an egg cell or a cell derived therefrom.

The present invention also relates to a method for the production of a transgenic mammal comprising introduction of a nucleic acid molecule or vector of the invention and optionally a template nucleic acid molecule into a germ cell, an embryonic cell or an egg or a cell derived therefrom. The invention also relates to transgenic animals obtained by said method.

Further, the present invention relates to transgenic mammalian cells, comprising stably integrated into the genome

- (a) a nucleic acid molecule encoding a polypeptide of the invention, which is linked to regulatory elements allowing transcription and/or expression of the nucleic acid molecule in mammalian cells and/or
- (b) a template nucleic acid molecule determined by the method of the invention which is linked to regulatory elements allowing transcription of said template nucleic acid molecule in mammalian cells.

In a further embodiment the invention relates to transgenic mammals comprising the above described mammalian cells.

In a further embodiment the present invention relates to a mammalian cell which contains stably integrated into the genome a nucleic acid molecule of the invention which is linked to regulatory elements allowing transcription of said nucleic acid molecule in mammalian cells wherein the presence of said nucleic acid molecule and/or the transcription and/or expression leads to reduction of the synthesis of the polypeptide of the invention in said cells.

In a preferred embodiment the reduction of the synthesis of the polypeptide of the invention in said cells is achieved by antisense, ribozyme and/or co-suppression effect. The present invention thus relates also to a transgenic mammal comprising such mammalian cells.

Preferably, the mammal referred to in the above embodiments is a human, a rat or a mouse.

Further embodiments and applications which also relate to the technology of transgenic mammals are discussed below with respect to transgenic plants. The person skilled in the art is able to apply, where appropriate, the technologies and applications discussed in connection with transgenic plants also to transgenic mammals.

The invention further relates to a transgenic plant cell comprising stably integrated into the genome

- (a) a nucleic acid molecule of the invention which is linked to regulatory elements allowing transcription and/or expression of the nucleic acid molecule in plant cells and/or
- (b) a template nucleic acid molecule determined by the method of the invention which is linked to regulatory elements allowing transcription of said template nucleic acid molecule in plant cells.

The nucleic acid molecules according to the invention are in particular useful for the genetic manipulation of plant cells in order to increase or decrease the RdRP activity in plants and by transferring the "RdRP-system" to organisms that lack a comparable mechanism to obtain plants with modified, preferably with improved or useful phenotypes.

For example, although a threefold increased concentration of the RdRP protein can be obtained by virus- or viroid-infection in tomato, the enzyme is still hardly detectable in those plants. Therefore it is expected that resistance of plants against RNA viruses can be improved by increasing the RdRP activity. For this purpose it is preferred to introduce at first a functional full-length RdRP cDNA copy into the genome of several crop plants. Regenerated transformants that have been screened for high RdRP activity have to be retransformed with a second construct to finally induce the RNA-mediated resistance against a particular virus. This transgene construct should provide *in vivo* transcripts which have to be homologous to a part of the virus RNA. In plants carrying both transgenes the improved resistance is based on the phenomenon that the increased RdRP activity leads to highly efficient production of "antisense" RNA. This would also include increased transcription of "antisense" RNAs from the nuclear-encoded virus-specific templates. These virus-specific "antisense" RNAs should be capable of inducing the sequence-specific RNA degradation process by hybridizing to the invading virus RNA.

The same gene silencing system could be applied to inactivate any endogenous gene in possibly all higher eukaryotes. In case that an efficient RdRP activity can be introduced into a particular organism it should be possible to down-regulate genes by re- or cotransforming with transgene constructs that are encoding the corresponding RdRP templates.

Thus, the present invention relates also to transgenic plant or mammalian cells which contain stably integrated into the genome a nucleic acid molecule according to the invention linked to regulatory elements which allow for expression of the nucleic acid molecule in plant or mammalian cells and wherein the nucleic acid molecule is foreign to the transgenic plant or

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mammalian cell and optionally a template nucleic acid molecule. For the meaning of the term "foreign", we refer to our definition provided herein above.

Further, the present invention also relates to transgenic plants or mammals comprising transgenic plant or mammalian cells according to the invention.

Furthermore, the invention relates to the transgenic plant cell which contains stably integrated into the genome a nucleic acid molecule according to the invention or part thereof, wherein the transcription and/or expression of the nucleic acid molecule or part thereof leads to reduction of the synthesis of a polypeptide having RdRP activity.

In a preferred embodiment, the reduction is achieved by an anti-sense, ribozyme and/or co-suppression effect.

The provision of the nucleic acid molecules according to the invention opens up the possibility to produce transgenic plant cells with a reduced level of the polypeptide as described above and, thus, inactivate antisense RNA production. Techniques how to achieve this are well known to the person skilled in the art. Experiments described in the prior art indicate that overexpressed transgenes as well as several tissue-specific or developmentally regulated endogenous plant genes can be post-transcriptionally silenced by the RdRP system. Therefore it should be possible to inactivate this putative protection mechanism by decreasing the RdRP activity. This inactivation could be performed, for example, by "antisensing" the RdRP mRNA, or by ribozymes, molecules which combine antisense and ribozyme functions, molecules which provide for cosuppression effects, or by tagging the plant RdRP gene. For both purposes the knowledge of the RdRP cDNA or RdRP gene sequence is a prerequisite. In order to post-transcriptionally silence the RdRP gene, real "antisense" transgene constructs have to be designed. It is easy to understand that a "sense" transgene

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construct does not make sense because the enzyme that is to be inactivated is just exactly the enzyme that can synthesize "antisense" RNA from "sense" RNA templates. The "antisense approach" to decrease the RdRP protein concentration could have the advantage of not completely destroying RdRP activity. In case the RdRP bears an essential part in healthy plants, the total loss of RdRP would be a lethal factor. As a function of the promoter driving a transgenic "antisense" construct or as a function of the transgene integration locus, plants could be isolated that show lower RdRP levels than wild-type plants. To analyse whether plants are able to survive without any RdRP activity, the RdRP gene can be knocked-out by gene tagging experiments. Foreign DNAs, as for example the T-DNAs of *Agrobacterium tumefaciens*, integrate into the plant genome at random loci. Provided that the sequence of the RdRP gene is known, plants can be screened for T-DNA insertions at the RdRP gene locus by PCR (Koes *et al.*, 1992). Plants in which successful tagging of the RdRP gene occurred, should survive because in a diploid plant only one allele is inactivated. In case RdRP-minus plants show normal growth, progeny homozygous for the insertion allele are obtained after selfing.

When using the antisense approach for reduction of the above described enzymatic activity in plant cells, the nucleic acid molecule encoding the antisense-RNA is preferably of homologous origin with respect to the plant species used for transformation. However, it is also possible to use heterologous nucleic acid molecules which display a high degree of homology to endogenously occurring nucleic acid molecules encoding a polypeptide with the respective enzymatic activity. In this case the homology is preferably higher than 80%, particularly higher than 90% and still more preferably higher than 95%.

For the expression of the nucleic acid molecules according to the invention in sense or antisense orientation in plant cells, the molecules are placed under the control of regulatory elements which ensure the expression in plant cells. These regulatory elements may be heterologous or homologous with respect

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to the nucleic acid molecule to be expressed as well with respect to the plant species to be transformed. In general, such regulatory elements comprise a promoter active in plant cells. To obtain expression in all tissues of a transgenic plant, preferably constitutive promoters are used, such as the 35 S promoter of CaMV (Odell et al., Nature 313 (1985), 810-812) or promoters of the polyubiquitin genes of maize (Christensen et al., Plant Mol. Biol. 18 (1982), 675-689). In order to achieve expression in specific tissues of a transgenic plant it is possible to use tissue specific promoters (see, e.g., Stockhaus et al., EMBO J. 8 (1989), 2245-2251). Known are also promoters which are specifically active in tubers of potatoes or in seeds of different plants species, such as maize, Vicia, wheat, barley etc. Inducible promoters may be used in order to be able to exactly control expression. An example for inducible promoters are the promoters of genes encoding heat shock proteins.

The regulatory elements may further comprise transcriptional and/or translational enhancers functional in plants cells.

Furthermore, the regulatory elements may include transcription termination signals, such as a poly-A signal, which lead to the addition of a poly A tail to the transcript which may improve its stability.

In the case that a nucleic acid molecule according to the invention is expressed in sense orientation it is in principle possible to modify the coding sequence in such a way that the polypeptide is located in any desired compartment of the plant cell. These include the endoplasmatic reticulum, the vacuole, the mitochondria, the plastides, the apoplast, the cytoplasm etc. Methods how to carry out these modifications and signal sequences ensuring localization in a desired compartment are well known to the person skilled in the art.

Methods for the introduction of foreign DNA into plants are also well known in the art. These include, for example, the transformation of plant cells or tissues with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* (EP-A 120 516; EP-A 116 718; Hoekema in: The Binary Plant Vector System,

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Offsetdrukkerij Kanters BV, Alblasserdam (1985), Chapter V; Fraley et al., Crit. Rev. Plant Sci. 4, 1-46 und An et al., EMBO J. 4 (1985), 277-287), the fusion of protoplasts, direct gene transfer (see, e.g., EP-A 164 575), injection, electroporation, biolistic methods like particle bombardment and other methods known in the art.

The transformation of most dicotyledonous plants is possible with the methods described above. But also for the transformation of monocotyledonous plants several successful transformation techniques have been developed. These include the transformation using biolistic methods (Wan and Lemaux, Plant Physiol. 104 (1994), 37-48; Vasil et al., Bio/Technology 11 (1993), 1553-1558), protoplast transformation, electroporation of partially permeabilized cells, introduction of DNA using glass fibers, etc.

The reduction of the synthesis of a polypeptide according to the invention in the transgenic plant cells results in an alteration in the modulation of gene expression. In transgenic plants comprising such cells this can lead to various physiological, developmental and/or morphological changes.

The present invention also relates to transgenic plants comprising the above-described transgenic plant cells.

The present invention also relates to cultured plant tissues comprising transgenic plant cells as described above which either show overexpression of a polypeptide according to the invention or a reduction in synthesis of such a polypeptide.

In yet another aspect the invention also relates to harvestable parts and to propagation material of the transgenic plants according to the invention which either contain transgenic plant cells producing a polypeptide according to the invention (and if an endogenous RdRP gene is present in the plant above the wild type level of RdRP in said plant) or which contain cells which show a

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reduced activity of the described polypeptide. Harvestable parts can be in principle any useful parts of a plant, for example, leaves, stems, fruit, seeds, roots etc.

Propagation material includes, for example, seeds, fruits, cuttings, seedlings, tubers, rootstocks etc.

In general, the plants which can be modified according to the invention and which either show overexpression of a polypeptide according to the invention or a reduction of the synthesis of such a polypeptide can be derived from any desired plant species. They can be monocotyledonous plants or dicotyledonous plants, preferably they belong to plant species of interest in agriculture, wood culture or horticulture interest, such as crop plants (e.g. maize, rice, barley, wheat, rye, oats etc.), potatoes, oil producing plants (e.g. oilseed rape, sunflower, pea nut, soy bean, etc.), cotton, sugar beet, sugar cane, leguminous plants (e.g. beans, peas etc.), wood producing plants, preferably trees, etc.

It is furthermore possible to use the nucleic acid molecules, vectors and/or polypeptides of the invention and, optionally, an appropriate RdRP template nucleic acid molecule which may be identified by the afore-described method for inhibiting expression of any desired gene by transferring the RdRP system to organisms that either lack a comparable mechanism or do not sufficiently express their own RdRP. Furthermore, the nucleic acid molecules, vectors, antibodies and/or antagonist/inhibitors of the present invention can be used for inhibiting RNA-directed RNA synthesis and thereby conferring stable heterologous gene expression in transgenic organisms.

Besides the above-described possibilities to use the nucleic acid molecules, vectors and polypeptides according to the invention for e.g. the genetic engineering of organisms and their use to identify homologous molecules, the described nucleic acid molecules may also be used for several other applications, for example, for the identification of nucleic acid molecules which

encode polypeptides which interact with the RdRP described above. This can be achieved by assays well known in the art, for example, by use of the so-called yeast "two-hybrid system". In this system the polypeptide encoded by the nucleic acid molecules according to the invention or a smaller part thereof is linked to the DNA-binding domain of the GAL4 transcription factor. A yeast strain expressing this fusion protein and comprising a lacZ reporter gene driven by an appropriate promoter, which is recognized by the GAL4 transcription factor, is transformed with a library of cDNAs which will express plant proteins fused to an activation domain. Thus, if a polypeptide encoded by one of the cDNAs is able to interact with the fusion protein comprising at least part of the RdRP polypeptide complex is able to direct expression of the reporter gene. In this way the nucleic acid molecules according to the invention and the encoded RdRP can be used to identify proteins interacting with the RdRP, such as protein kinases, transcription factors and the like.

Other methods for identifying proteins which interact with the proteins according to the invention or nucleic acid molecules encoding such molecules are, for example, the *in vitro* screening with the phage display system as well as filter binding assays.

~~Description of the Figures -~~

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1

15 µg of genomic tomato DNA (cv. Rentita) were cut with EcoR1 (lane 1), BamH1 (lane 2), Hind3 (lane 3), Xba1 (lane 5), and Hae3 (lane 6), respectively, (5U/µg) and electrophorized in a 13 x 18 cm 0.8% TEA-agarosegel. The DNA was then vacuum-blotted onto a nylon membrane and hybridized against a random primed ³²P-labeled RdRP-specific cDNA fragment as a probe.

The membrane was autoradiographed for 16 h at -80°C using a BIOMAX™ MR film (Kodak) and a QuantalIII-intensifying screen (Du Pont).

Lane 4: molecular weight standard, λ-DNA cut with Pst 1.

Figure 2

15 µg of genomic DNA isolated from potato (lane 1), tobacco (lane 2), and the two tomato cultivars "St. Pierre" (lane 4) and "Rentita" (lane 5) were cut with Hind3 (5U/µg) and electrophorized in a 13 x 18 cm 0.8% TEA-agarosegel. The DNA was then vacuum-blotted onto a nylon membrane and hybridized against a random primed ³²P-labeled RdRP-specific cDNA fragment as a probe.

The membrane was at first autoradiographed for 16 h to visualize lane 1, 4, and 5 and then for 72 h to visualize lane 2 at -80°C using a BIOMAX™ MR film (Kodak) and a QuantalIII-intensifying screen (Du Pont).

Lane 4: molecular weight standard, λ-DNA cut with Pst 1.

Figure 3

15 µg of total RNA isolated from PSTVd-infected (+) and viroid-free (-) tomato plants (cv. Rutgers and cv. Basket Pak, respectively), were denaturated by glyoxalation and electrophorized in a 13 x 18 cm 1.5% phosphate-buffered

agarose gel. The RNA was then capillar-blotted onto a nylon membrane and hybridized against a random primed ^{32}P -labeled RdRP-specific cDNA fragment as a probe.

The membrane was autoradiographed for 16 h at -80°C using a BIOMAXTM MR film (Kodak) and a Quantalix-intensifying screen (Du Pont).

Figure 4

1 μl aliquots of elution fractions from 4 different protein purification columns (lane 2 and 3: Q Sepharose FF, lane 4: Poly(A) Sepharose, lane 5: Poly(U) Sepharose, and lane 6: Heparin Sepharose) that contained the highest RdRP activity were applied onto SDS-PAGE (Phast system, Pharmacia) and were then subjected to Western analysis. Immuno-detection was performed with the RdRP-specific antibody $\text{A}_{\text{P}431}$.

Lane M: molecular weight protein standard (205, 116, 97, and 58 kDa proteins).

Lane 1: 1 μl aliquot of the protein extract that was subsequently loaded onto the Q Sepharose FF column.

Numbers in parentheses are corresponding to the numbers of two Q Sepharose FF elution fractions.

Figure 5

1 μl aliquots of the Sephadex G-200 gel filtration fractions [lane 2: retention volume fraction (rvf) 12, lane 3: rvf 14, lane 4: rvf 15, lane 5: rvf 17, and lane 6: rvf 19] were applied onto SDS-PAGE (Phast system, Pharmacia) and were then subjected to Western analysis. Immuno-detection was performed with the RdRP-specific antibody $\text{A}_{\text{P}431}$.

Lane 1: 1 μl aliquot of the protein extract that was subjected onto the column (ON).

Figure 6

Design of RdRP-specific PCR-Primers.

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EXAMPLES

~~Description of the invention~~

Example 1: RdRP purification

The concentration of plant-encoded RdRP in non-infected and apparently healthy plants is rather low. From 1 kg of apical tomato leaves corresponding to about 1000 tomato plants only about 10 to 20 µg of a protein preparation having RdRP activity could be isolated (Schiebel *et al.*, 1993a). This low concentration is also reflected by the fact that the RdRP preparation from tomato leaves had to be concentrated ~100,000-fold to obtain at least an electrophoretic homogeneous protein preparation (Schiebel *et al.*, 1993a). Micro-sequencing of protein preparations purified according to the method of Schiebel *et al.* (1993a) gave no reliable sequence information.

Thus, a new purification method had to be applied which was performed as follows. RdRP was solubilized from freshly harvested apical leaves of tomato plants (*Lycopersicon esculentum*, cultivar 'Rentita'). The plants that were grown under semi-controlled greenhouse conditions (25°C, 15-h photoperiod) were systemically infected (Tabler and Sanger, 1985) with the PSTVd type strain (Gross *et al.* 1978) at the two leaf stage i.e. about 4 weeks after sowing (Tabler and Sanger, 1984). Only the young and still unexpanded leaves from the shoot tips (about 1 g per plant) were harvested three to four weeks after inoculation.

Portions of 100 g each were homogenized with 500 ml of buffer A (50 mM Tris-acetate, pH 7.4, 10 mM K-acetate, 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.5 mM PMSF) in a Waring type blender (Braunmixer MX 32) for 2 minutes at position 3. The homogenate was strained through 135 µm nylon sieve cloth (Nytal, Schweizer Seidengazefabrik, CH-9425 Thal SG/Swiss) and the retentate was rehomogenized with 220 ml of buffer A. The combined filtrates from 200g of leaf material were centrifuged at 1.000 x g for 15 minutes (Beckman Rotor J6B). The supernatant was mixed with 0.3 volumes of glycerol 86% and the mixture centrifuged at 30.000 x g for 1 h in a Beckman type 19 rotor. The supernatant containing the "soluble RdRP"

(RdRP-s) and the majority of TNTase was used for ammonium sulfate precipitation.

The 30.000 x g pellet containing RdRP was resuspended in 100 ml of buffer B (50 mM Tris-acetate, pH 8.2, 50 mM K-acetate, 1 mM EDTA, 25% (v/v) glycerol (Ph.Eur.III), 10 mM 2-mercaptoethanol, 0.5 mM PMSF) using a Dounce homogenizer. The suspension was slowly stirred for 30 minutes and centrifuged for 50 min at 40,000 rpm (146.000 x g) in a Beckman rotor 50.2 Ti. The supernatant represents extract E1. The pellet was reextracted twice with 0.5 ml/g buffer B resulting in extract E2. Extracts E1 and E2 containing the 'membrane-bound RdRP' (RdRP-b) were shockfrozen in solid CO₂ and stored at -70°C for loading onto a DEAE-Sephadex column.

The 30.000 x g supernatant was mixed with the same volume of 3.95 M ammonium sulfate solution. After stirring for one hour at 4°C, the suspension was centrifuged at 10,000 x g for 30 min. The pellet was dissolved with 40 ml of buffer C (25 mM Tris-acetate, pH 8.2, 1 mM EDTA, 20% glycerol, 3 mM 2-mercaptoethanol), dialyzed over night against 1 liter of buffer C, cleared by centrifugation at 10,000 x g for 30 min, shockfrozen in solid CO₂ and kept at -70°C for chromatography.

Both, supernatant and extracts from the pellet were assayed for RdRP activity as follows: The assay for RdRP activity is based on the incorporation of radioactively labeled 5'-nucleoside monophosphates from the corresponding [α -³²P]NTPs into transcription products of a given RNA template. This assay is not RdRP-specific because the ubiquitous terminal nucleotidyl transferases (TNTase) are able to terminally incorporate radioactive ribonucleotides into polymeric RNA products. Such TNTases are RNA uridylyl-transferase (Zabel et al., 1981) (EC 2.7.7.52), polyadenylyl-transferase (Edmonds, 1982) (EC.2.7.7.19) or other TNTases (Boege, 1982) which usually contaminate RdRP preparations. No specific direct assays are available, as yet, that allow the unambiguous discrimination between the true template-directed transcription by RdRP and the terminal addition of nucleotides to the designated template RNA by TNTases. Consequently,

RdRP and contaminating TNTases had to be separated from each other before their individual activities can be quantitated.

One unit of enzyme activity is defined as the amount of enzyme which catalyzes the incorporation of 1 nmol 5'-uridine monophosphate into high molecular weight RNA within 30 min. If not stated otherwise the assay was performed in prelubricated 1.7-ml test tubes (Sorenson Bioscience, Inc. MultiTM Liquid Handling Products, Salt Lake City, Utah, USA) in a final volume of 25 μ l containing 50 mM Tris-acetate (pH 7.8), 10 mM Mg-acetate, 2 mM dithioerythritol, 0.3 mM each of ATP, CTP, GTP, 0.02 mM [α -³²P]UTP (adjusted to 0.5 Ci/mMol), 1 μ g TMV RNA, 0.01% Tween 20, and 2 or 5 μ l of RNA polymerase preparation. Reactions were initiated by adding the enzymes. After 30 min of incubation at 37°C, the reaction tubes were transferred to ice. After mixing with 15 μ l of 13 mM UTP, 10 μ l of water was added as a wash solution and the tube contents were spotted on Whatman 3MM paper strips (2 x 10 cm) before performance of descending chromatography in 2 M Na-acetate, pH 5.2/ethanol = 1/1. The radioactivity of the RNA products remaining at the origin of the chromatogram was determined by liquid scintillation counting (6 ml cocktail rotiszint eco plus, Roth) and used for the calculation of enzyme activity. Under the given conditions and 5 μ l RdRP/assay a concentration of one unit per milliliter corresponded to 4800 cpm (Schiebel *et al.*, 1993b). This assay was used throughout the examples unless otherwise stated.

The supernatant contained about 5 U per 150 g of leaves whereas the corresponding pellet contained about 1.4 U of extractable RdRP.

All RdRP preparations were first chromatographed on DEAE-Sepharose which removed nucleic acids, most of the pigments and an inhibitor of RdRP activity.

The extracts containing RdRP-b from the membrane-rich pellet or the ammonium sulfate-fractionated 30,000 x g supernatant were loaded onto a column of DEAE-Sepharose Fast Flow (FPLC system, Pharmacia; 5 cm x 10 cm) with a flow rate of 6 to 15 cm/h (2 - 5 ml/min) using a peristaltic pump.

After eluting non-adsorbed material with about 70 ml of buffer C, a linear gradient of 0.0 to 0.7 M ammonium acetate in buffer C was applied with a flow rate of 3 to 12 cm/h (1 to 4 ml/min) depending on the pressure limit of about 0.5 MPa. The volume of the gradient was 500 ml up to 0.5 M salt and 100 ml for the higher salt concentrations. Fractions of 20 ml were collected and those fractions containing the main enzyme activity were pooled, shockfrozen and stored.

The enzyme preparation from the DEAE-Sephacrose chromatography containing about 0.27 M ammonium acetate was diluted with one volume of buffer D (20 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 1.5 mM DTE, and 0.012% Tween 20) and loaded onto 3 ml poly(U)Sephacrose 4B (Pharmacia) in a 1 x 3.7 cm column (FPLC system, Pharmacia; C10/10) equilibrated with buffer D. A total volume of 120 ml was loaded with a flow rate of 1 ml/min. After elution of nonadsorbed proteins with buffer D a linear gradient of 30 ml from 0.0 to 1 M NaCl in buffer D was applied and fractions of 1.5 ml were collected. Those fractions containing enzyme activity were pooled, shockfrozen and stored.

In order to concentrate the RdRP containing eluate from the first poly(U)-Sephacrose chromatography the corresponding fractions were diluted with two volumes of buffer D and loaded on 0.4 ml poly(U)-Sephacrose 4B in a 0.5 x 1.9 cm column (SMART system, Pharmacia; HR 5/2) with a flow rate of about 0.1 ml/min using a peristaltic pump. After elution of non-adsorbed proteins with buffer D the column was installed into the Smart system. Elution was performed by sodium chloride increasing in a linear gradient up to 1.5 M. The flow rate during elution was 0.05 ml/min. Fractions of 0.1 ml were collected. Those fractions containing enzyme activity were analyzed by SDS PAGE and pooled resulting in the partially purified RdRP preparation.

Pools from several chromatographic runs were collected. The proteins exhibiting a RdRP activity of 20 U in 4 ml desorption buffer (about 1 M NaCl in buffer D) were concentrated and concomitantly freed from salt by deoxycholate trichloroacetic acid precipitation (Bensadoun and Weinstein, 1976) in prelubricated 1.7-ml test-tubes. Insulin (25 µg/ml) was used as

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coprecipitant, centrifugation was performed in a swinging bucket rotor at 11.000 rpm for 30 minutes. The resulting pellets were washed with ethanol at -20°C and used for analytical SDS PAGE (Phast system, Pharmacia). The estimated amount of RdRP protein was about 5 µg.

Example 2: Microsequencing of the RdRP proteins

The precipitated protein was prepared for SDS PAGE, applied onto a 7% polyacrylamide gel (Laemmli) and stained with coomassie Brilliant Blue R250 (Sambrook *et al.*, 1989). The 128 kDa band was excised and cleaved in the gel according to Eckerskorn and Lottspeich (1989) except that instead of trypsin, endoprotease LysC (Boehringer Mannheim) with an enzyme:protein ratio of 1:10 (w/w) was used. The peptides separated by reversed phase HPLC were sequenced using a 492A amino acid sequencer (Applied Biosystems) according to the manufacturers instructions.

Example 3: Isolation of the RdRP cDNA

Micro-sequencing of the RdRP protein resulted in only four definite AS-sequences from which the corresponding nucleic acid sequence could be deduced. In Fig. 6 the sequence of two of the four peptides and the sequence of four of the corresponding synthetic oligonucleotides that had been designed for PCR-experiments are presented.

In order to perform PCR with the RdRP-specific primers, cDNA had been synthesized from tomato mRNA. For mRNA isolation 20g of young leaves were harvested from potato spindle tuber viroid- (PSTVd) infected "Rutgers" tomato plants that were grown in the greenhouse (see above). The plant material was immediately frozen in liquid nitrogen, and total RNA was isolated as described by Logemann *et al.* (1987). From 5mg of total RNA, polyA+ RNA was isolated using the 'PolyATtract mRNA Isolation System I' (Promega) following the manufacturers instructions. Subsequently 1µg of the purified polyA+ RNA was subjected to the 'cDNA Synthesis Kit' (Boehringer Mannheim) and cDNA was synthesized according to the manufacturers

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instructions using an oligo (dT)₁₅ primer. The reaction was stopped by phenol-extraction and 1 µl of the sample was diluted 1:100 with TE-buffer.

PCR was performed with 1 µl of the diluted cDNA and using the primer-pair A (RdRP/8H1-RdRP/15R1; 0.1 nmol each) and B (RdRP/15H1-RdRP/8R1; 0.1 nmol each), respectively. Amplifications were assayed in a 100 µl reaction-mix containing 10 µl of 10x Assay Buffer (Eurogentec), 10 µl of dNTPs (2 nmol/µl), and 1 µl of EuroTaq polymerase (4U/µl) (Eurogentec). The samples were transferred to a 'Crocodile II Thermocycler' block (Appligene) and 30 cycles were started (1' 95°C, 1' 55°C, and 1' 72°C). After separation on a 0.8% TBE-agarose gel (Sambrook *et al.*, 1989), analysis of the PCR products revealed that a single DNA fragment of about 800 bp in size had been amplified with primer pair B. This fragment (RdRP₈₀₀) was eluted from the agarose gel (QIAquick Gel Extraction Kit, Qiagen) and cloned (Sambrook *et al.*, 1989) into the T/A-type PCR cloning vector pTPCR (Wassenegger *et al.*, 1994) which is similar to the plasmid pCRTMII that is delivered with the cloningTM Kit (Invitrogen).

The sequence of the RdRP₈₀₀ was determined on an automatic sequencer (ALFexpress, Pharmacia Biotech) using the 'Cy5 AutoRead Sequencing Kit' (Pharmacia Biotech) and following the manufacturers sequencing procedure. The precise length of RdRP₈₀₀ was 833bp comprising an ORF that corresponds to 277 amino acid residues (AS) given in SEQ ID NO:3 which correspond to amino acid 700 to amino acid 917 of the amino acid sequence of SEQ ID No:2.

Two custom ZAP EXPRESSTM EcoRI cDNA libraries (Stratagene) had been established from polyA⁺ RNA which had been isolated as described above from young leaves of the tomato cultivars 'Rutgers' and 'Basket Pak'. From five to 10 µg of the purified polyA⁺-enriched RNA cDNA libraries were constructed (Stratagene). The size-fractionated oligo(dT)-primed cDNAs (>500 bp) were ligated *via* EcoRI adaptors into the Lambda ZAP bearing pBK-CMV phagemid vector. This system has a cloning capacity of 12 kb and allows *in vivo* excision of the cloned fragments. However, the "Rutgers"- and

"Basket Pak"-specific cDNA libraries produced by Stratagene had a representative size of 1.800 bp and 3.5×10^8 primary plaques.

Both libraries were screened three times by plaque hybridization following the instruction manual of the Stratagene ZAP EXPRESSTM EcoRI Library with the ³²P-labeled RdRP₈₀₀-DNA fragment using the 'Random Primed DNA Labelling Kit' (Boehringer Mannheim). Altogether 23 recombinant plasmid DNAs that had been detected with the radioactive probe were excised *in vivo* from the phages and finally introduced into the XLORTM *E.coli* strain according to the Stratagene ZAP EXPRESSTM EcoRI Library instruction manual. Characterization of the plasmid inserts revealed that none of them corresponded to the minimum size of 3.000 bp which could be expected from a 120kDa protein. The largest hybridizing EcoRI cDNA fragment (RdRP24) was about 2.300 bp in length, and it contained the entire 3'-part of the RdRP. In order to obtain the missing 5'-region of the RdRP cDNA, the 'rapid amplification of 5'-cDNA ends' (RACE) was performed. This PCR technique allows the amplification of cDNA by using one appropriate gene specific reverse primer (GSP) for which the sequence information about the 5'-end is not required.

Oligo-dT-primed cDNA was synthesized as described (see above) using polyA⁺ RNA that has been isolated from PSTVd-infected tomato plants. According to the "MarathonTM cDNA Amplification Kit" (Clontech) a particular adaptor was ligated to both ends of the produced double-stranded cDNA. The subsequent PCR amplification was performed with a 1:100 dilution of the 'adaptored' cDNA (1µl/reaction) using an oligonucleotide that was complementary to the adaptor sequence (adaptor primer, AP) as a forward primer. As reverse primers, the following three different GSPs (GSP400, GSP420, and GSP1200) complementary to the known RdRP24-specific cDNA sequence were designed:

GSP400: 5'-CAT AAC GAA TCT GGA AAG CAG ATG G-3' (SEQ ID NO:4)

GSP420: 5'-GAT GAA TCC GGA TCA ACA CCC ACA C-3' (SEQ ID NO:5)

123456789101112131415161718192021222324252627282930313233343536373839404142434445464748495051525354555657585960616263646566676869707172737475767778798081828384858687888990919293949596979899100

GSP1200:5'-GGG TGC TGG AGG ATA TTC CAT CGG C-3' (SEQ ID NO:6)

Thermal cycle parameters as dependent on the annealing temperature of the GSP were as follows:

Program 1 (T_m: 60-65°C) used for GSP400 (T_m=57.9°C) and GSP420 (T_m=62.1°C):

94°C for 1 min; 30 cycles: 94°C/30 sec, 60°C/30 sec, 68°C/4 min

Program 2 (T_m: 65-70°C) used for GSP1200 (T_m=66°C):

94°C for 1 min; 30 cycles: 94°C/30 sec, 68°C/4 min

In case of the GSP400-specific RACE reaction, a major product of about 1.900 bp was amplified. In consideration of a 400 bp long overlap with RdRP24 this 1.900 bp fragment contained a RdRP-specific 5'-end sequence of about 1.500 bp. In contrast to the AP/GSP400-specific PCR product, agarose/EtBr gel analysis of the AP/GSP420-specific PCR products revealed that shorter DNA fragments had been amplified. Because GSP420 was complementary to the RdRP24 sequence at position +420, the appearance of PCR products that were smaller than 1.900 bp in size indicated that predominantly inappropriate cDNAs had been synthesized. The reactions performed with the AP/GSP1200 resulted in fragments of 2.700 bp in length. After subtraction of 1.200 bp which are provided by the overlap between GSP1200 and RdRP24, the remaining fragment again indicated that the RdRP24 cDNA clone has to be extended by 1.500 bp to give a full-length RdRP-specific cDNA of about 3.800 bp in size.

Several inserts of different length were obtained after cloning of the gel-purified AP/GSP400-specific PCR products into pTPCR (see above). Sequence analysis (see above) of the largest RACE clones had shown that the cloned insert most likely comprised the complete 5'-end of the RdRP-specific cDNA. Computer-supported sequence analysis (DNASIS, Pharmacia) of a fusion of the AP/GSP400-specific RACE product with the RdRP24 sequence revealed the entire RdRP cDNA sequence (SEQ ID NO:1) with an Open Reading Frame of 1114 amino acids and a calculated molecular weight

of the RdRP protein of about 127 kDa which is in good agreement with the experimental values that had been determined by SDS-PAGE (128 kD) and sucrose gradient centrifugation (119 kD) (Schlebel *et al.*, 1993a).

In order to physically generate of a full-length cDNA clone, many efforts were made to connect the RdRP24 fragment with the AP/GSP400-specific PCR product. Although a suitable unique *AspI* restriction site was found to be present in the overlapping area of both subclones, cloning of the gel purified DNA fragments into different vectors and transformation into different *E. coli* strains failed. Because none of these attempts was successful, the PCR technique was again utilized to produce the entire RdRP cDNA.

For this purpose forward primers had been designed that were specific to region 30 (P127BamA) and to region 140 (P127BamB) of the 5'-nontranslated region of the RdRP sequence (see above). As a reverse primer, an oligonucleotide (P127Bgl) complementary to the 3'-nontranslated region (position 3630, see above) was synthesized.

P127Bam1: 5'-CTT CAC CAG GGA TCC ACT CAT CAC TCC CCT CAA G-3' (SEQ ID NO:7)

P127Bam2: 5'-GCA TAA CTT CAG GGG GGA TCC AGT TGG TGT TAG C-3' (SEQ ID NO:8)

P127BGL: 5'-GCA GCT TCA TGC AGA TCT AAA GAC AAA AGG TAG TC-3' (SEQ ID NO:9)

To enhance the cloning efficiency these oligonucleotides contained a unique restriction site, i. e. a *Bam*HI sites in case of the forward primers, and a *Bgl*II site in case of the reverse primer. PCRs were performed with tomato cDNA as described above, running the thermo cycler program 1 and using P127Bgl ($T_m=65.6^\circ\text{C}$) in combination with either P127BamA ($T_m=72.5^\circ\text{C}$) or with P127BamB ($T_m=71.1^\circ\text{C}$). As a result, major products of the expected sizes ranging between 3.500 and 3.600 bp were obtained. After double digestion with *Bam*HI/*Bgl*II the PCR products were cloned into the plasmid vector pT3T7 (Boehringer Mannheim) that had been previously cut with *Bam*HI.

Sequence analysis of four independent clones revealed that all RdRP-specific inserts represented the entire RdRP cDNA. Unfortunately it turned out that they all contained several substitutions when compared with each other, and also when compared with the original chimeric sequence. Although most of these mutations were found to be "still", because only the third position of a codon triplett was affected, others could lead to an unwanted translational stop. The error rate of the EuroTaq polymerase (Eurogentec) is obviously too high to amplify 3.500 bp fragments without nucleotide substitutions. Therefore, another PCR approach was performed under reaction conditions as described above but using the Expand™ High Fidelity PCR System (Boehringer Mannheim). This system utilizes a mixture of the Taq and the Pwo DNA polymerase, the later enzyme possesses an efficient 3'-5' proofreading ability. RdRP-specific products were again amplified, and after sequencing of the corresponding cloned cDNAs it was demonstrated that a lower error rate had occurred within the sequences. Finally, one clone (RdRP/HF) only contained two "still" nucleotide substitutions. The mutations within the RdRP/HF sequence are presented in figure 3/1. The RdRP/HF 5'-BamHI site, and the BglII site which is located at the 3'-end of RdRP and which is destroyed upon cloning a BglII fragment into a BamHI site are also shown.

Table 1

	BamHI				(BglII/BamHI)
	:	10	1995	2235	: 3575
	:	*	*	*	*:
RdRP/HF	GGATCC	ACTC-//	-GATTACTCTC-//	-CGACTTGCGG-//	-ATAAGAGATC C
	30		2020	2260	3600
	*	*	*	*	* *
RdRP cDNA	GGATTG	ACTC-//	-GATTACGCTC-//	-CGATTGCGG-//	-ATAAGAAAAC T

Table 1: Partial alignment of the RdRP/HF and the RdRP cDNA sequences
 The nucleotide substitutions detected within the coding region of the RdRP/HF sequence are underlined. Letters set in italics are indicating the cloning sites.

Example 4: Southern analysis of tomato genomic DNA

Tomato genomic DNA was analysed to determine the copy number and the genome organization of the RdRP gene. The knowledge of the RdRP cDNA sequence and the computer-supported compilation (DNASIS, Pharmacia) of its restriction map allows the detection of gene-exons and gene-introns by Southern hybridization of genomic DNA.

Genomic DNA was extracted from tomato leaves (20g) according to the procedure of Bedbrook (1981). 15µg of purified DNA were cut with EcoRI, BamHI, HindIII, XbaI, and HaeIII (all enzymes were from Boehringer Mannheim), respectively, and electrophorized on a 0.8% TAE agarose gel (Sambrook *et al.*, 1989). After the denaturation and renaturation of the agarose gel, the DNA was transferred (Vacuum Blotter, Appligene) to positively charged Nylon Plus membrane (Qiagen), and finally UV_{312nm}-crosslinked (0.3J/cm²). Hybridization of the Southern blot against radom primed ³²P-labeled RdRP24 DNA (see above) was performed as described by Amasino (1986).

The autoradiograph of the Southern blot (exposed for 16 hours) is shown in figure 1. The endonucleases EcoRI and BamHI do not cut within the RdRP cDNA and most likely there is also no restriction site within the RdRP gene. Therefore the single band of about 10.000 bp (lane 1 and lane 2), respectively, indicates that the RdRP gene exists as a single copy gene within tomato genome. This assumption is supported by the occurrence of only a single hybridizing fragment in lane 4. Because XbaI cuts only once within the RdRP cDNA, the second XbaI site must be located in the flanking genomic DNA close to the RdRP gene sequence. As a consequence, if the plant

A more detailed analysis of the tomato RdRP gene by characterization of clones that had been isolated from two custom ZAP EXPRESS™ EcoRI cDNA genomic libraries (Stratagene) is in progress. So far, the entire sequence of the coding region could be confirmed, and the existence of at least three introns could also be demonstrated (data not shown).

Of particular interest is the examination of the distribution of the RdRP gene over different plant species. Therefore Southern hybridization was performed as described above, but now HindIII-restricted genomic DNA of the two different tomato cultivars 'Rutgers' and 'St. Pierre, as well as nuclear DNA of potato and tobacco was analysed. The autoradiograph of the Southern blot (exposed for 16 h lane 1,3, and 4; exposed for 72 h lane 2) revealed that the RdRP gene is detectable in all four genomes (Fig. 2). The strong cross-hybridization of the potato DNA with the tomato-specific probe (lane 1) is not unexpected because potato is a plant that is closely related to tomato. The weak 'tobacco signals' (lane 2) might be due to the fact that tobacco is more distantly related to tomato. In addition, the tobacco genome is much more

Example 6: Northern analysis of total tomato RNA

The autoradiograph of the Northern analysis which was exposed for 16 h shows that the RdRP gene is transcribed, and moreover It shows that transcription is induced upon PSTVd-Infection (Fig 3, compare lane 1 and 3 with lane 2 and 4). This result is in good agreement with the observation that the amount of RdRP protein is also increased in viroid infected tomato plants.

In order to examine whether the RdRP cDNA encoded protein (C-protein) is identical with the isolated enzyme, four different C-protein-specific antibodies had been produced (Eurogentec). From the entire AS sequence of the C-protein the following peptides had been synthesized and were chosen for immunisation of rabbits (Eurogentec):

P431: ASKTFDRRKDAEAI (SEQ ID NO:11)

P432: EQYDGYLKGRQPPKSPS (SEQ ID NO:12)

P433: VFPQKGKRPHNEC (SEQ ID NO:13)

The specific reaction of each antiserum with the RdRP protein isolated from the tomato leaf tissue was tested by Western blotting (Sambrook *et al.*, 1989). The four antisera were able to immunologically detect a protein at the position of the RdRP activity (data not shown). Subsequently the two most sensitive antisera were applied onto a peptide/antigen matrix column for affinity purification (Eurogentec). The purified P431 antiserum (A_{P431}) was used in all experiments described below.

Specific immunological detection of the active RdRP protein with A_{P431} was analysed with elution fractions from four different chromatographic runs. 1 µl aliquots of those fractions that contained the highest RdRP activity were applied onto SDS-PAGE (Phast system, Pharmacia) and were then subjected to Western analysis (Fig. 4). As shown in figure 4 the intensity of the A_{P431}-specific signal correlates with the enzyme activity in that only those fractions reacted with A_{P431} that also contained active RdRP. As a control the protein sample that was run in lane 1 originates from the resuspended '30.000 x g precipitate' (see above) that was loaded on the Q Sepharose FF column. The additional signal that is visible in lane 2 and 3 was demonstrated to be due to reaction of a pre-serum-specific antibody with a protein that is eluted from the Q-Sepharose FF column together with the RdRP protein.

Additional evidence of the averment that the RdRP cDNA encodes the RdRP enzyme came from a gel filtration experiment (Fig. 5). The Sephadex G-200 gel system is used to separated proteins according to their molecular weight. This size fractionation procedure was originally conceived to further purify the plant RdRP protein. Surprisingly enough the retention volume for the elution of the tomato enzyme corresponded to a protein with a size of less than 65 kDa. As shown in figure 4 the highest RdRP activity was found in fraction 15 and not in fraction 13 as expected. But with the aid of SDS-PAGE analysis it was demonstrated that fraction 15 contained a 128 kDa protein and moreover this protein was detected by A_{P431} (Fig 4., Immuno-blot, lane 15).

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The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and any nucleic acid molecules, proteins, constructs or antibodies which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

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